Evaluation of Sowing Methods to Determine the Role of Hypocotyl Lift in Longleaf Pine Seedling Development

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Abstract

Sonderegger pine (*Pinus x sondereggeri* H.H. Chapm.) is a hybrid pine species of loblolly pine (*Pinus taeda* L.) and longleaf pine (*Pinus palustris* Mill.) that is culled when noticed during seedling processing and packing in the nursery. Early indication of the hybrid has been theorized as the absence of a seed wing stub or hypocotyl lift off of the growing medium surface, although neither of these theories has been proven. Two trials were conducted to determine the role of container cell color, growing medium depth in the cells, and the presence of a seed wing stub on longleaf pine hypocotyl lift. Seedlings grown in black container cells had increased hypocotyl lift and tendency for reductions in root-collar diameter growth regardless of seed wing stub presence. Genetic testing indicated that both wingless and winged seeds were true longleaf pine. This paper was presented at the Joint Annual Meeting of the Southern Forest Nursery Association and the Northeast Forest and Conservation Nursery Association (Pensacola, FL, July 17–19, 2018).

Introduction

Sonderegger pine (*Pinus x sondereggeri* H.H. Chapm.) occurs naturally where longleaf pine (*Pinus palustris* Mill.) and loblolly pine (*Pinus taeda* L.) are in close proximity and have the potential to cross-pollinate and hybridize. Traditionally, this hybrid species is considered to be of low quality, with poor bole formation and limb distortion (Wakeley 1954). In addition, the loblolly pine genes in the hybrid cause the terminal bud of Sonderegger pine to become extended, often resulting in the bud being in direct line with flames from prescribed burning, which is required for true longleaf pine ecosystems to thrive (Jose et al. 2006). Because of these observable qualities, Sonderegger pine seedlings are generally culled from the nursery in an effort to prevent them from being erroneously planted as pure longleaf pine.

Sonderegger pines are typically culled when noticed by nursery workers during lifting. The hybrid seedlings exhibit bud elongation among true longleaf pine seedlings (figure 1), which conversely, have no bud elongation and instead have resting buds near the medium surface in containers. In describing longleaf pine germination phases, Boyer (1990) stated that "newly germinated seedlings have virtually no aboveground hypocotyl, and the cotyledons are close to the ground line." A theory exists that the elongation of the longleaf pine hypocotyl or the lifting of the cotyledons from the medium surface are signs that hybridization has occurred in that

Figure 1. The red circle indicates a Sonderegger pine seedling growing among longleaf pine seedlings in a container nursery. (Photo by Paul Jackson 2018)

seedling. Often, however, true longleaf pine seedlings exhibit a slight hypocotyl lift in the nursery, and to our knowledge, there have been no research trials conducted to determine the cause of the lift and whether it is truly indicative of hybridization.

Another theory proposed to detect Sonderegger pine seedlings in the nursery is the absence of a wing stub on longleaf pine seed. Wakeley (1954) stated that “the seed wings of all southern pines except longleaf can be rubbed or broken cleanly from the dry seeds. No way of completely dewinging longleaf seed in bulk has been discovered; commercial ‘dewinging’ merely reduces the wings to stubs.” Wakeley goes on to state that “when the wing of a seed of any southern pine except longleaf is thoroughly moistened, the two curved prongs which attach the wing to the seed straighten out within a few seconds and the seed falls away at a touch. Advantage is sometimes taken of this fact in dewinging species other than longleaf.” Thus, it was theorized that longleaf pine seeds missing a wing stub may be expressing this loblolly pine characteristic and are hybrid Sonderegger pine.

Three longleaf pine trials were conducted at Louisiana Tech University in the 2018 growing season to test the two theories. The first trial’s objective was to determine if container cell color and growing-medium depth in the container cells affect hypocotyl lift. This objective centers on the premise that the lack of light reaching deeper into the cell, the way light is reflected due to container color, or the heat generated around the seedling in black containers may cause hypocotyls to lift off of the medium. Germination of longleaf pine seed in darkness can cause hypocotyl extension to lengths of 1 in (McLemore 1967), thus, the idea that shallow-filled container cells may reduce light availability and subsequently contribute to hypocotyl lift. Even though containers are filled with growing medium at the nursery using mechanized equipment, the chance of a cell not reaching operational capacity exists, especially cells on the edges. The second trial’s objective was to determine if container cell color, medium depth, and the presence of a seed wing stub affected hypocotyl lift. The third trial’s objective was to compare DNA between winged and wingless longleaf seeds to determine if any tested positive for the loblolly marker.

Materials and Methods

Trial One

Longleaf pine seeds from a Florida source and with intact wing stubs were soaked in an aerated water bath for 12 hours and stratified in a refrigerator for 9 days before being sown into Ray Leach Cone-tainer™ cells (RL98 Stubby, Stuewe and Sons, Inc., Tangent, OR) on March 16, 2018, at Louisiana Tech University (figure 2). A peat moss based Pro-Mix® growing medium was used. Two container colors (white and black) and two fill levels (operational level or two-thirds of operational level) were evaluated in the trial for a total of four treatments (figures 3 and 4). Filling container cells to normal levels and to two-thirds of normal levels (referred to from this point as lower levels) resulted in root plugs that were approximately 5-in and 3-in (12.7- and 7.6-cm) deep, respectively. Each tray of 49 cells served as a replication, and there were three

Figure 2. Longleaf pine seeds were sown into white and black container cells. (Photo by Paul Jackson 2018)

Figure 3. Black container cells filled with growing medium to normal operational levels. (Photo by Paul Jackson 2018)

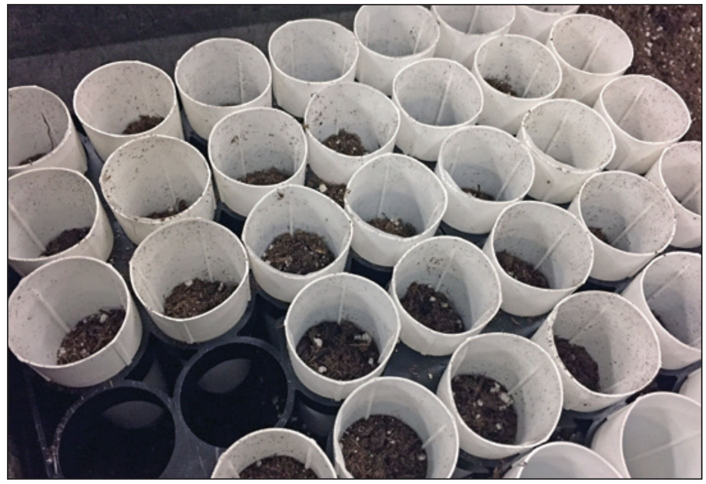

Figure 4. White container cells filled to two-thirds capacity with growing medium. (Photo by Paul Jackson 2018)

trays per treatment for a total of 588 container cells sown with longleaf pine seed. Trays were placed on greenhouse benches under misters that supplied water for 45 seconds every hour from 6:00 am to 6:00 pm. On April 9, 2018, the trays were moved outside to growing benches located in full sun and kept there for the duration of the trial. Seedlings were measured 98 days after sowing. Root-collar diameter (RCD), hypocotyl lift defined as the length of the hypocotyl lifted off of the growing medium (figure 5), and the length of the entire hypocotyl from the base of the cotyledons to the first divergence of a lateral root (figure 6) were measured on each seedling.

Trial Two

A second trial was installed on May 4, 2018, involving the same materials and methods as described previously, with the difference being that a third factor (longleaf pine seeds with or without an intact wing stub, figure 7) was added for a total of eight treatments. The same measurements as described previously were recorded 108 days after sowing on August 20, 2018.

Data Analyses

For both Trials One and Two, an analysis of variance was conducted using a General Linear Model (GLM) and multiple comparisons of means were conducted using Duncan's Multiple Range Test using SAS statistical software (9th ed., SAS Institute, Cary, NC).

Figure 5. Longleaf pine seedling showing hypocotyl lift off of the growing medium. This example is a seedling grown in a black container cell filled to two-thirds capacity with growing medium. Seedlings grown in cells filled with less medium averaged a root-plug depth of approximately 3 in (7.6 cm). (Photo by Paul Jackson 2018)

Trial Three

To determine if wingless seeds have a genetic predisposition to experience hypocotyl lift compared to winged seeds, deoxyribonucleic acid (DNA) from 20 winged and 20 wingless longleaf pine seed were analyzed and compared by using two chloroplast

Figure 6. A measurement of the entire hypocotyl on a seedling from the base of the cotyledons to the first lateral root (Photo by Paul Jackson 2018)

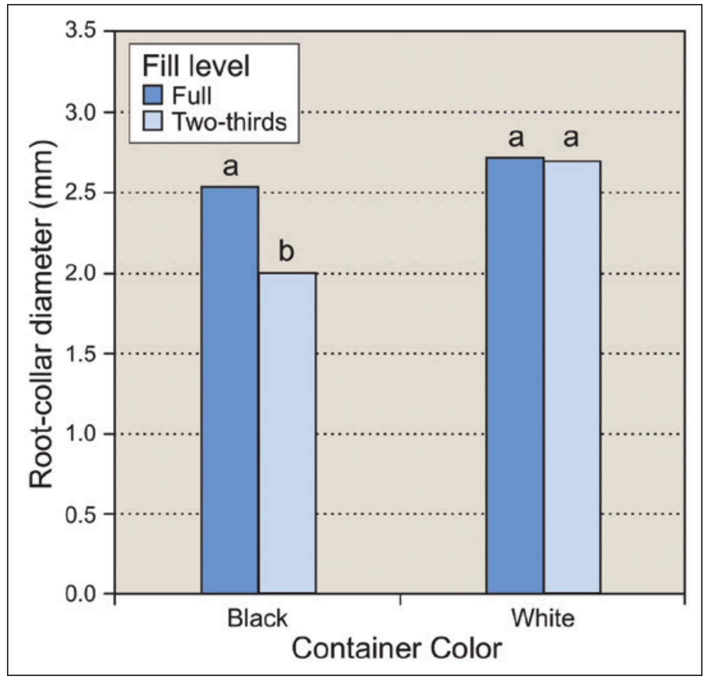

Figure 8. Mean root-collar diameter (RCD) was significantly smaller for seedling grown in black containers with less growing medium (two-thirds capacity) compared with all other treatments.

Figure 7. Longleaf pine seed with an intact wing stub (left) and missing the entire wing (right). (Photo by Paul Jackson 2018)

DNA markers with a set of specific primers for each (one for longleaf pine and one for loblolly pine). The DNA extracted from each seed was performed using the Qiagen DNeasy® Plant Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. The DNA from known longleaf pine and loblolly pine were included in the PCR amplification to serve as positive controls in evaluating the seed samples. Amplification of DNA was performed in 10 µl PCR reaction in an Eppendorf Mastercycler® Pro PCR machine (Eppendorf AG Hamburg, Germany). Gel electrophoresis was performed to examine amplified products by loading 5 µl PCR products on 1-percent agarose gels. The agarose was stained with ethidium bromide after 20 minutes of electrophoresis, and the resulting bands were visualized under ultraviolet (UV) illumination to confirm positive or negative amplifications. A band indicates positive amplification, while no band indicates negative. A positive with the longleaf marker identifies a sample as a longleaf pine, while a negative with longleaf marker indicates the sample is not a longleaf pine. Similarly, a positive with the loblolly marker identifies a sample as a loblolly or a Sonderegger pine, while a negative with the loblolly marker indicates the sample is not a loblolly or a Sonderegger pine. Therefore, if a sample is longleaf-marker positive, it is not a Sonderegger hybrid.

Results

Trial One

Longleaf pine seedlings grown in black container cells filled with lower levels of medium had significantly smaller RCDs compared with all other treatments (table 1, figure 8). Seedlings grown in black cells had more hypocotyl lift compared to those grown in white cells, and seedlings grown in cells filled with less medium experienced more hypocotyl lift compared to cells filled to operational levels (figures 5 and 9). Among all treatments, there were no differences in total hypocotyl length (figure 9).

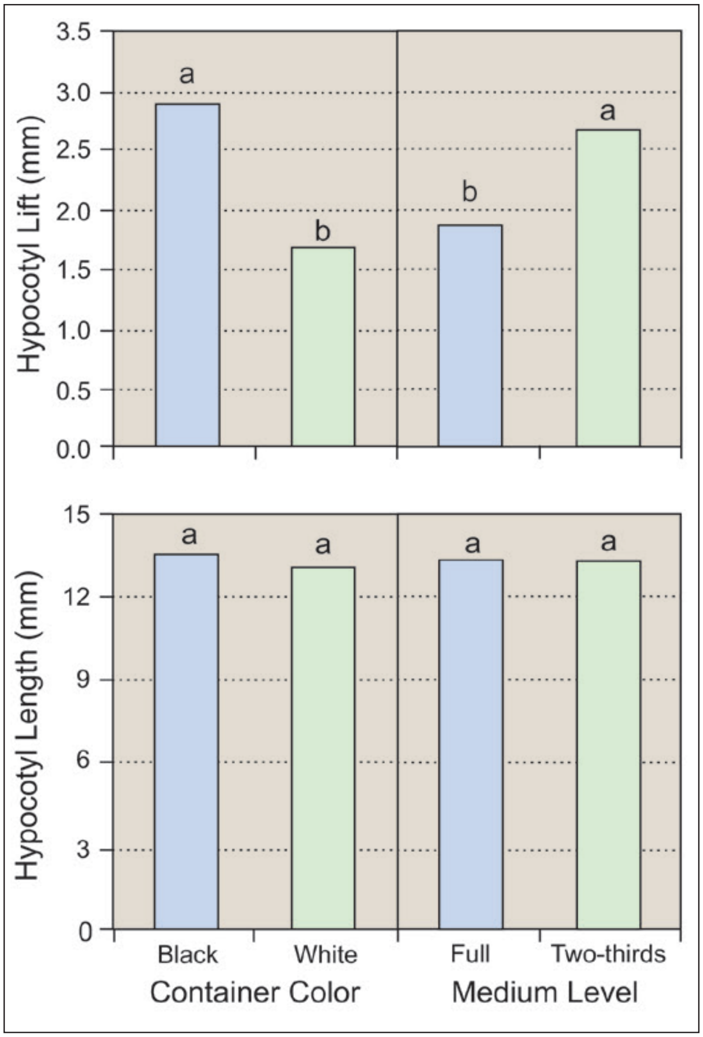

Figure 9. In Trial 1, mean hypocotyl lift off of the growing medium differed between longleaf pine seedlings grown in black and white container and between seedlings grown in normal operational levels of growing medium or two-thirds of normal levels. Mean hypocotyl length, however, was not influenced by either treatment factor. Means for each treatment factor were compared using Duncan's Multiple Range Test.

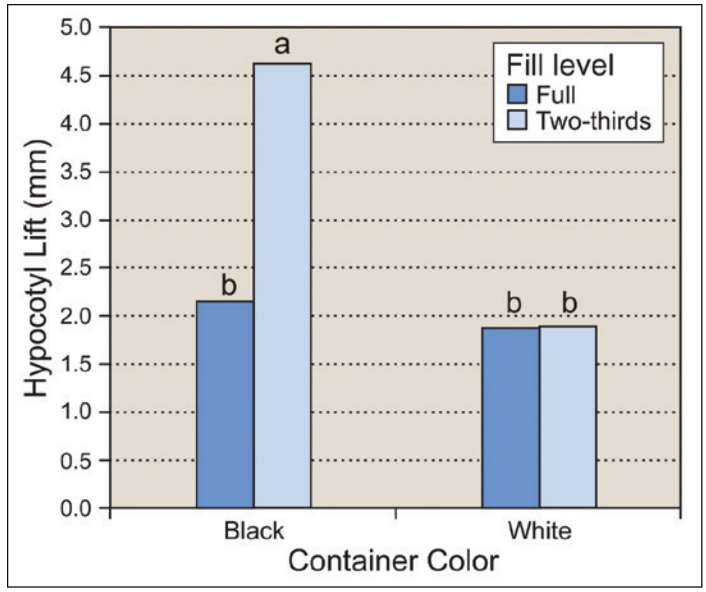

Figure 10. Mean hypocotyl lift off of the growing medium in Trial 2 had a significant interaction between container color and amount of growing medium.

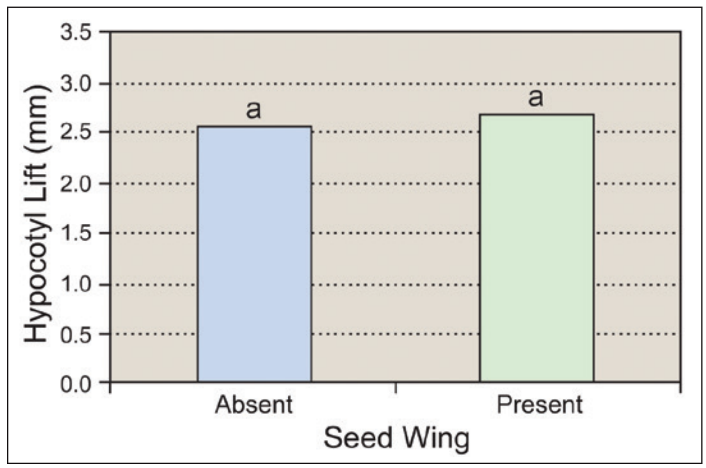

Figure 11. Mean hypocotyl lift did not differ between seedlings grown from seed with and without wing stubs in Trial 2 based on Duncan's Multiple Range Test.

Table 1. Probability values for treatment main effects and interactions for Trial 1.

Treatment	Root-collar diameter (mm)	Hypocotyl lift (mm)	Total hypocotyl length (mm)
Main Effects (P > F)			
Color	0.0003	0.0211	0.1014
Medium Depth	0.0058	0.0978	0.8309
Color*Medium	0.0080	0.2570	0.3018

Trial Two

Longleaf pine seedlings grown in black cells with less growing medium had increased hypocotyl lift for both winged and wingless seeds (table 2, figure 10). The amount of hypocotyl lift that occurred between winged and wingless was similar (figure 11).

Hypocotyl length in seedlings grown from wingless seeds was shorter compared to those on seedlings grown from winged seeds (table 2, figure 12). Seedlings grown in black cells had smaller RCDs compared to seedlings grown in white cells (table 2, figure 13).

Trial Three

All 20 wingless and 20 winged longleaf pine seeds tested positive with the longleaf pine marker along with the known longleaf positive control, while all 20 winged and 20 wingless DNA samples evaluated tested negative with the loblolly pine marker.

Table 2. Probability values for treatment main effects interactions for Trial 2.

Treatment	Root-collar diameter (mm)	Hypocotyl lift (mm)	Total hypocotyl length (mm)
	Main Effects (P > F)		
Color	0.0218	0.0016	0.1452
Medium Depth	0.6358	0.0064	0.2648
Seed	0.7740	0.7553	0.0008
Color*Medium	0.1385	0.0071	0.4830
Seed*Medium	0.1250	0.9082	0.2888
Seed*Color	0.5625	0.5582	0.6093
Seed*Color*Medium	0.2074	0.9603	0.2343

Discussion

With none of the wingless longleaf pine seeds testing positive to the loblolly pine DNA marker and no seedling differences found in Trial Two between seed types, the theory of wingless longleaf pine seeds being an indicator of hybrid Sonderegger pine does not hold true. In another trial conducted in 2016, out of 343 seedlings grown from winged longleaf pine seed and 392 seedlings grown from wingless longleaf pine seed, only three seedlings developed into true Sonderegger pines: one from a winged seed and two from wingless seeds (unpublished data). The occurrence of wingless seeds in a seedlot may relate more to how seeds from certain sources respond to seed extraction and processing.

In the nursery, slight hypocotyl lift is often observed on longleaf pine seedlings. The elongation of the hypocotyl either ceases and normal longleaf pine development occurs or the elongation of the hypocotyl continues and a Sonderegger pine seedling develops. In both trials, seedlings in all treatments had some level of hypocotyl lift. Seedlings grown in black container cells had more hypocotyl lift (both trials) and smaller RCD (Trial One only) compared with those grown in white container cells. In a study with red maple (*Acer rubrum* L.) and bush beans (*Phaseolus vulgaris* L.), black containers generated more heat and caused substrate temperatures to increase (Markham et al. 2011). Neither substrate temperature nor ambient temperature at the container cell level was recorded in these trials though we speculate that higher temperatures in the black cells stimulated hypocotyl lift and caused reductions in RCD growth.

Total hypocotyl length did not differ among treatments, but hypocotyl lift off of the growing medium tended to be more when container cells were filled to two-thirds capacity. After testing hypocotyl extension in known longleaf pine, loblolly pine, and hybrid seeds, Brown (1964) stated that "the intensity of light and varying temperature conditions during the period of hypocotyl elongation has little effect on final hypocotyl length in longleaf pine, whereas either light or temperature greatly influences the rate and duration of hypocotyl elongation in loblolly pine. The hybrid population lies between these extremes." Brown's assertion may have given more insight to the findings in these trials had there been an indication that any of the seeds (winged or wingless) compared positively to the loblolly

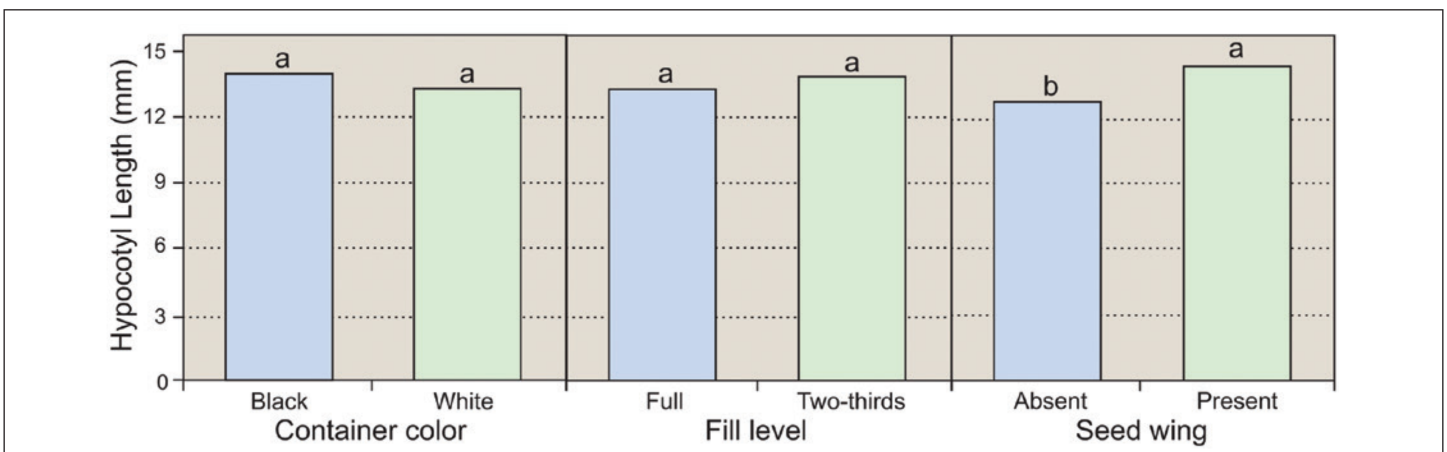

Figure 12. In Trial 2, mean total hypocotyl length of longleaf pine seedlings was unaffected by container color or the amount of growing medium in the container but was significantly longer for seedlings grown from winged seeds compared with wingless seeds. Means for each treatment factor with the same letter are not significantly different based on Duncan's Multiple Range Test.

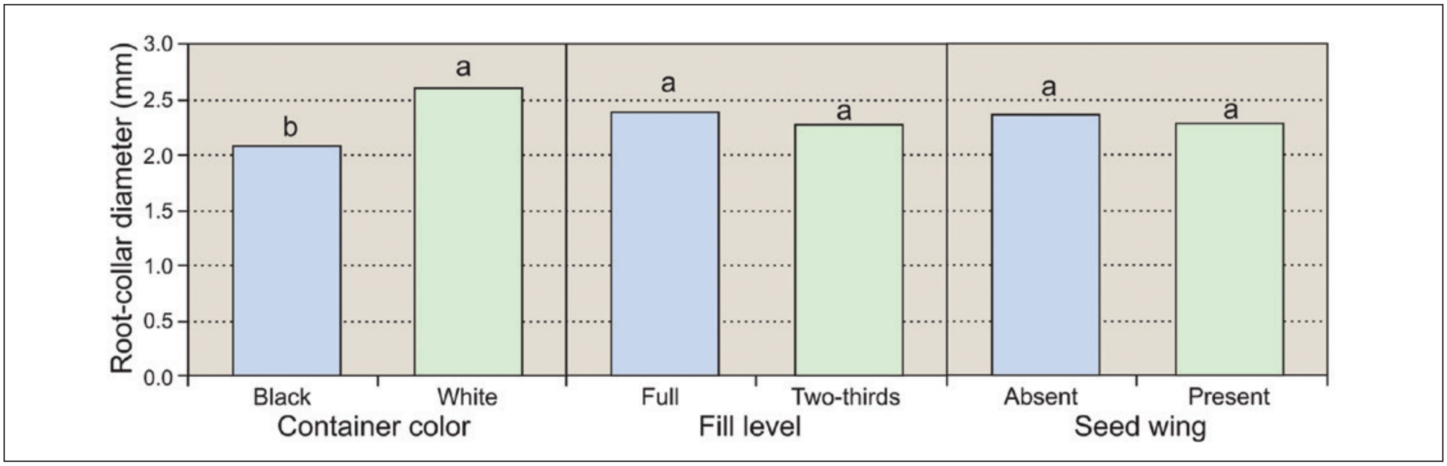

Figure 13. Root-collar diameter (RCD) of longleaf pine seedlings in Trial 2 was significantly influenced by container color but was unaffected by the amount of growing medium or the presence of wings on the seed. Means for each treatment factor with the same letter are not significantly different based on Duncan's Multiple Range Test.

pine genetic marker in the DNA comparison test. Based on our DNA comparisons, all were likely true longleaf pine. Thus, hypocotyl extension off of the growing medium in cells filled to two-thirds capacity is most likely a result of environmental factors such as light availability or light reflection in the container cell.

Future Research Direction and Considerations

Longleaf pine seedlings are traditionally grown in black containers in nurseries across the South. Future research trials to evaluate potential indicators of Sonderegger pine should include measurements of substrate temperature, ambient temperature near the growing-medium surface, and light intensity. These measurements could then be related to morphological data observed during seedling development. Trials could also be developed to administer differing levels of light and/or expose seedlings to certain temperatures in controlled settings. Testing other species such as loblolly pine or slash pine (*Pinus elliottii* Engelm.) would also be useful in comparison to longleaf pine results.

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